Calcite Microcrystals in the Pineal Gland of the Human Brain: Second Harmonic Generators and Possible Piezoelectric Transducers

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ABSTRACT

A new form of biomineralization in the pineal gland of the human brain has been studied. It consists of small crystals that are less than 20 μm in length and that are completely distinct from the often-observed mulberry-type hydroxyapatite concretions. Cubic, hexagonal and cylindrical morphologies have been identified using scanning electron microscopy. Energy dispersive spectroscopy, selected-area electron diffraction and near infrared Raman spectroscopy established that the crystals were calcite. Experiments at the European Synchrotron Radiation Facility (ESRF) to study the biomineralization showed the presence of sulfur originating from both heteropolysaccharides and amino acids. Other studies at the ESRF furnished information on the complex texture crystallization of the calcite. With the exception of the otoconia structure of the inner ear, this is the only known non-pathological occurrence of calcite in the human body. The calcite microcrystals are believed to be responsible for the previously observed second harmonic generation in pineal tissue sections. There is a strong possibility that the complex twinned structure of the crystals may lower their symmetry and permit the existence of a piezoelectric effect.

Index Terms — **Microcrystals, calcite, piezoelectricity, second harmonic generation, scanning electron microscopy, Raman spectroscopy, sulfur, crystal texture, synchrotron.**

1 INTRODUCTION

THE pineal gland is a neuroendocrine transducer that converts a neural signal into an endocrine output [1]. It secretes a number of hormones, the most important of which is melatonin which synchronizes the physiological Circadian rhythm [2]. Pineal calcifications have been found in numerous animals and in humans and are the only crystalline forms known in the pineal gland [3]. Two major forms of pineal crystalline structures have been observed: (i) polycrystalline complexes with dimensions of the order of hundreds of micrometers, often called mulberry-like structures or concretions, and (ii) well-defined microcrystals having long dimensions as large as 20 μm. Weak second harmonic generation (SHG) has been observed in pineal tissue samples [4]. Although the concretions have been studied extensively, no experiments have been done previously on the microcrystals. In this research, the microcrystals were studied by a large number of techniques: scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), high-resolution transmission electron microscopy (HRTEM), selected area electron-diffraction (SAED) and near infrared Raman spectroscopy. Experiments were made at the European Synchrotron Radiation Facility (ESRF) to study the roles of heteropolysaccharides and amino acids in the formation of the microcrystals and their structural texture.

2 CHARACTERIZATION OF THE MICROCRYSTALS

A total of 20 human pineal glands from subjects ranging in age from 15 to 68 years were supplied by the Institute of Pathology of the Soroka Medical Centre in Beer Sheva, Israel and by the Anatomopathology Service of the CHU Michalon in Grenoble, France. The glands were fixed in 10% formalin. The microcrystals were isolated from the pineal glands using a dilute sodium hypochlorite solution following a procedure developed by Weiner and Price [5].

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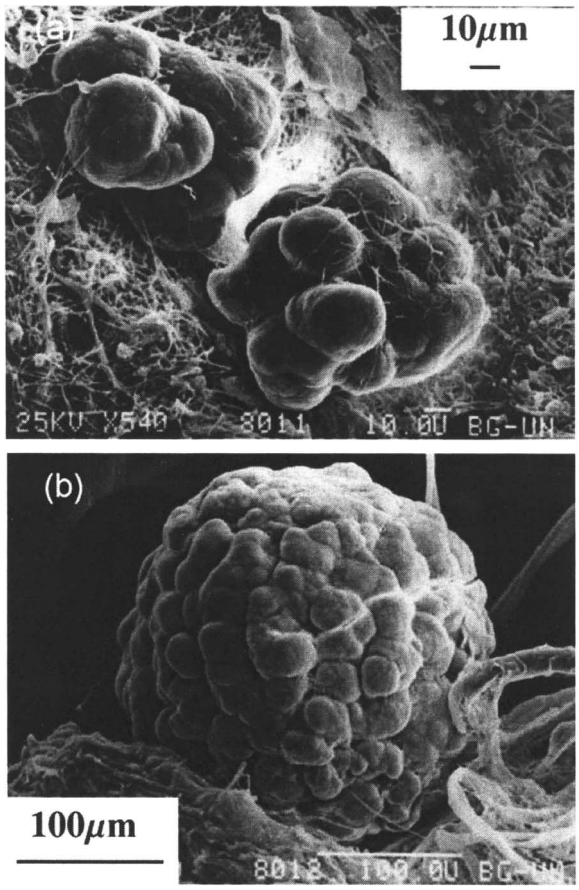

Figure 1. SEM photos of mulberry-like concretions in cryofractured pineal tissue. a, small concretions with lobes; b, large conglomerate.

It should be emphasized that, at no point, did any of the samples come into contact with solutions containing calcium ions. Microcrystals were found in every gland in quantities ranging from 100 to 300 crystals/mm^3 of gland. No attempt was made to correlate the quantity of crystals with either the age of the subject or pathological details.

SEM samples were collected on transmission electron microscopy grids and analyzed with a JEOL JSM 5600 SEM. Microanalysis studies were performed with a NORAN EDS Analyzing System. As a reference, SEM photographs were taken of the mulberry-like concretions. Two general sizes were observed as shown in Figure 1. Their outer structure was similar to those observed by others [6,7]. More relevant to the present project were the single microcrystals. Three different shapes of crystals were observed, cubic, hexagonal and cylindrical as shown in Figure 2. The length dimensions of the crystals varied from 2 to about 20 μm. The most common morphology was a cylindrical body with sharp extremities. These comprised about 95% of the samples observed. Edges were usually very sharp and the body surfaces were very rough. The NORAN EDS Analyzing System coupled to the SEM was used to determine the composition of the crystals. The principal elements identified were calcium, carbon and

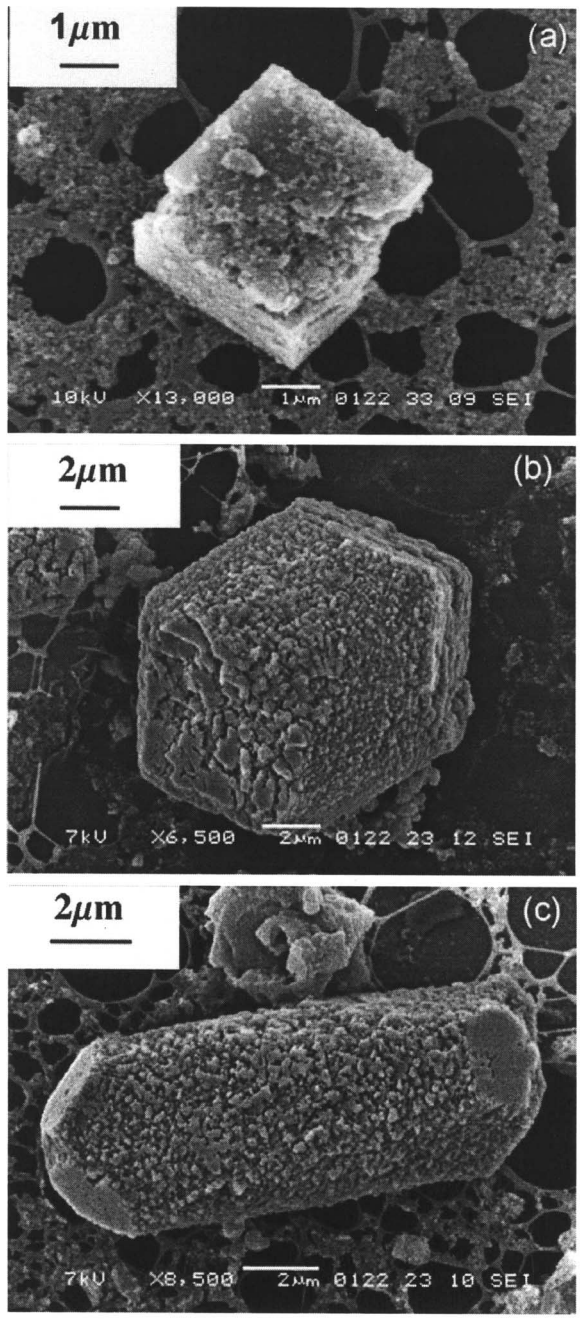

Figure 2. SEM of isolated pineal microcrystals on a Formvar-covered TEM grid. Three different crystal shapes were observed. a, cubic; b, hexagonal; c, cylindrical.

oxygen with less than 0.5 wt% each of silicon, aluminum, sodium and magnesium. No phosphorus was found. Among biominerals containing calcium, carbon and oxygen, only calcite (calcium carbonate) and calcium oxalate are potential candidates

Because the microcrystals were too thick for HRTEM observation, they were first crushed between two glass slides. They were studied in a JEOL-2010 transmission electron microscope equipped with an analytical ISIS system for energy dispersive X-ray spectroscopy (EDS). A

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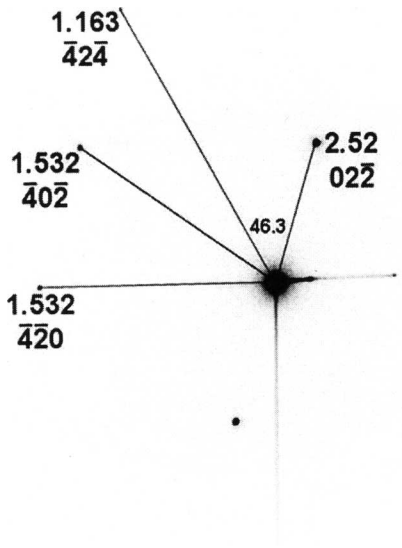

Figure 3. Indexed SAED pattern from fragment of microcrystal.

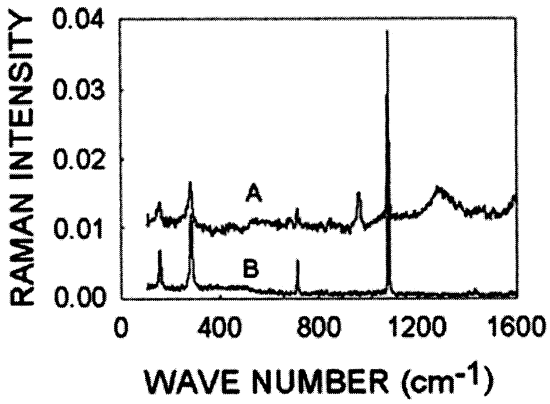

Figure 4. Raman spectra of pineal gland microcrystals (A) and pure calcite powder (B) .

typical diffraction pattern is shown in Figure 3. The electron diffraction patterns taken from these particles were indexed in terms of a hexagonal unit cell with lattice parameters $a = 4.989$ nm, $c = 17.062$ nm and $\alpha = 120°$. The dimensions are the same as those of calcite.

Near infrared Raman spectra of isolated crystals and of pure calcite were obtained with a Bruker IFS 66 FTIR spectrometer equipped with an FRA 106 Raman module and a Ramanscope microscope. The spectral resolution was 2 cm^{-1}. The agreement of the peaks was excellent (Figure 4), confirming the identification of the crystals as calcite. The additional peaks at 962 and 1283 cm^{-1} may have come from another chemical substance present in the crystal, such as a protein.

Lang et al. [4] have observed SHG in dried thin slices of pineal gland. The observed SHG signal intensities were about 10^{-3} times that of a standard urea powder. In the present study, SHG measurements were made to determine the origin of the previous observations. The only crystalline materials in the pineal tissue were hydroxyapatite concretions and calcite microcrystals. Both hydroxyapatite and calcite are centrosymmetric and would not be expected to show SHG by the usual dipolar mechanism [8]. However, calcite has been shown to exhibit second harmonic generation, albeit far weaker than in SHG active non-centrosymmetric crystals [9–11]. The SHG in calcite is quadrupolar in nature and phase-matchable, and is preferentially along a specific crystal direction due to birefringence. For a powdered sample of pure calcite, we measured an SHG intensity that was 4 orders of magnitude weaker than a urea powder standard. We were unable to detect SHG in either pure hydroxyapatite powder ($< 5 \times 10^{-6}$ times that of urea) nor in the large hydroxyapatite pineal concretions. SHG could not be detected in a small sample of isolated pineal microcrystals, due to the small number of microcrystals in the sample and their lack of proper orientation with respect to the incident laser beam. However, the similarity of the intensity of the SHG in pure calcite to that observed in earlier work on pineal tissue samples [4] and the absence of SHG in the large concretions show that the calcite microcrystals were the source of the SHG.

3 SULFUR IN THE CALCITE MICROCRYSTALS

Preliminary experiments to determine the mechanism of formation of the calcite microcrystals were carried out at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. Calcite biomineralization occurs in an organic matrix in species such as corals, sea urchin spine and sponge spicules [12]. Sulfur can be found in amino acids and heteropolysaccharides [13], two of the major types of compounds found in organic matrices. Sulfur in the amino acids, cysteine and methionine has a valence of -2 in a disulfide-type bond. Among the heteropolysaccharides are the glycosaminoglycans that contain sulfur in a sulfate group as chondroitin sulfate. The objective of our experiments was to locate and analyze the organic matrix in the microcrystals. Soft x-ray synchrotron radiation can be used to access the K-absorption edges of elements of major interest in the biological sciences, specifically sulfur in this case. The studies were carried out on the X-ray Microscopy Beamline (ID21). A schematic drawing of ID21 is shown in Figure 5. The x-ray beam was focused using Fresnel zone plates and raster-scanned over the crystals with a 0.25×0.25 μm^2 resolution. A Si-111 monochromator and a solid-state Ge energy-dispersive detector were used to observe the fluorescence in the range of energies from 2.450 keV to 2.530 keV. Because the test specimen was sufficiently thin, a transmission signal could be measured by means of a silicon photodiode.

The fluorescence spectra of cysteine, methionine and chondroitin sulfate were first obtained (Figure 6). Peaks at energies of about 2.472 and 2.473 keV were observed

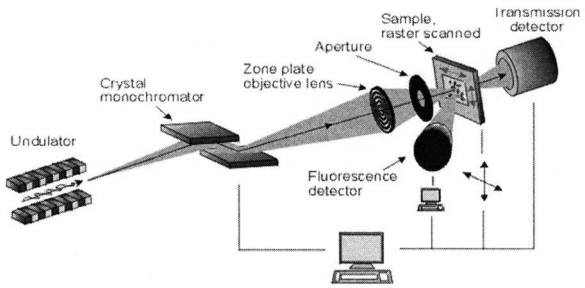

Figure 5. Layout of the X-ray Microscopy Beamline (ID21) at ESRF (Drawing courtesy of ESRF).

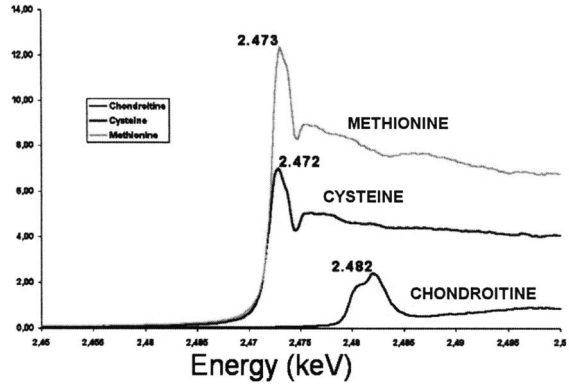

Figure 6. Fluorescence spectra of methionine, cysteine and chondroitine.

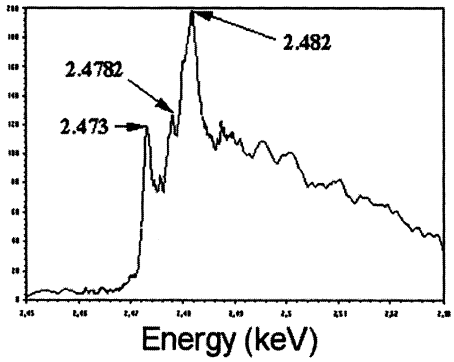

Figure 7. Fluorescence spectrum of the microcrystal from 2.45 keV to 2.53 keV.

for the disulfide bonding in cysteine and methionine, respectively, and a peak at about 2.482 keV was observed for the sulfate bonding in chondroitine sulfate. Then calcite crystals were deposited on a 4-μm thick plastic layer (SpexCertiprep©) and glued to the sample holder. A reticule made of a 25-μm tungsten wire was mounted beside the sample to enable the crystals to be located more easily. The samples were mounted in the x-ray microscope sample holder and analyzed under high vacuum ($\sim$1 Pa). A typical fluorescence spectrum is shown in Figure 7. The peak at 2.473 keV is characteristic of sulfur in amino acids and that at 2.482 keV for sulfur in heteropolysaccharides.

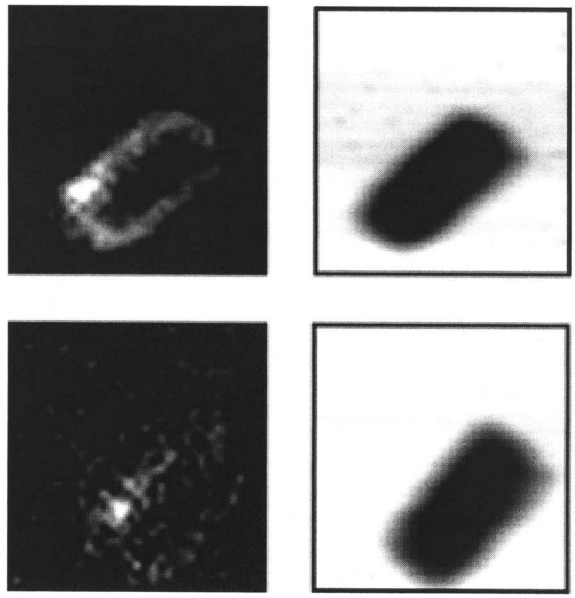

Figure 8. Location of sulfur compounds. Fluorescence channel (upper and lower left) and transmission channel (upper and lower right). Measurements at sulfate energy (2.482 keV) (upper left and right). Measurements at sulfide energy (2.473 keV) (lower left and right).

The fluorescence channel and the transmission channel results for the two energies are shown in Figure 8. The two sulfur compounds were present in the same regions of the crystal but the signal from the amino acids was weaker. The sodium hypochlorite solution used in the isolation of the crystals oxidized most of the organic materials resulting in weak fluorescence from the residual sugars. A different crystal isolation technique will be used in future studies.

4 TEXTURES OF THE MICROCRYSTALS

The texture of the calcite crystals was studied at ESRF on the Microfocus Beamline (ID13). This facility provides a monochromatic focal spot of about 20×20 μm^2 by using a channel-cut Si-111 monochromator and an ellipsoidal mirror. This beam is further reduced in size by glass capillary optics to give a 2-μm beam at the exit. A CCD detector is used. A schematic drawing of the beamline is shown in Figure 9. The sample was mounted on a glass fiber which was held by a goniometer head. The goniometer head was placed on a translation stage that could be raster scanned in 1-μm steps both horizontally and vertically or rotated in 0.1° steps about a vertical axis. A high-magnification microscope could be moved on a micropositioner in order to align the sample in front of the glass capillary. The glass fiber, goniometer, microscope and the glass capillary that focused the x-ray beam are shown in the photograph in Figure 10.

A microcrystal was placed on the glass fiber and it adhered due to a static electric charge. Diffraction patterns

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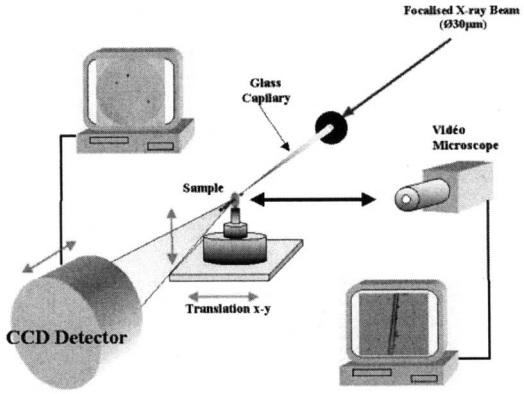

Figure 9. Layout of the Microfocus Beamline (ID13) at ESRF (Reproduced with permission from [22]). The image of the crystals on a glass fiber are shown on the monitor connected to the video microscope. The diffraction pattern in Figure 11 is shown on the monitor connected to the CCD detector.

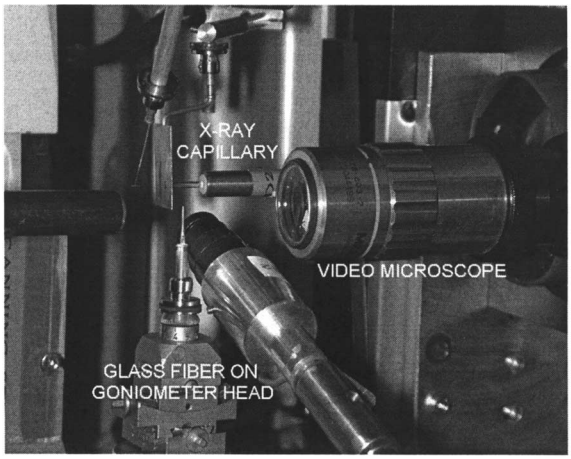

Figure 10. Photograph showing the glass fiber, goniometer, microscope and x-ray focusing glass capillary.

were obtained with a 1 μm resolution in horizontal and vertical scans and a rotational resolution of 0.1°. A typical diffraction pattern is shown in Figure 11. The upper reflection and the lower-righthand reflection are (113) and (024), respectively. Only a few reflections appeared because of the narrow angle covered by the detector. Figures 12 (left) and 12 (right) show the intensity of the (113) and (024) reflections for a 15 μm vertical scan and a rotation of 3°. The intensity of the reflections varied markedly with very slight translation or rotation of the sample. If the sample had been a uniform single crystal, all of the reflections would have had the same intensity. The variation in intensities indicates that adjacent microregions of the crystal are slightly out of crystallographic alignment with one another. A complete mathematical analysis of the data is presently in progress.

5 DISCUSSION

Most of the prior research concerned investigations of the large mulberry-form concretions. Recently, two studies described the presence of small geometric shapes associated with the concretion globules [14,15], but detailed investigations were not carried out. The technique developed here for isolation of the crystals gave us access to large enough quantities for a study of this new mineral structure which we have tentatively named "myeloconia" ("brain dust" in Greek). In contrast to the concretions with their globular and often conglomerate structure, the microcrystals have characteristic cubic, hexagonal or cylindrical crystalline morphologies. The concretions have sizes as large as a few millimeters whereas the microcrystals are not more than 20 μm in length. The microcrystals have the appearance of an agglomerate of small crystals but the overall morphology shows that those structures are

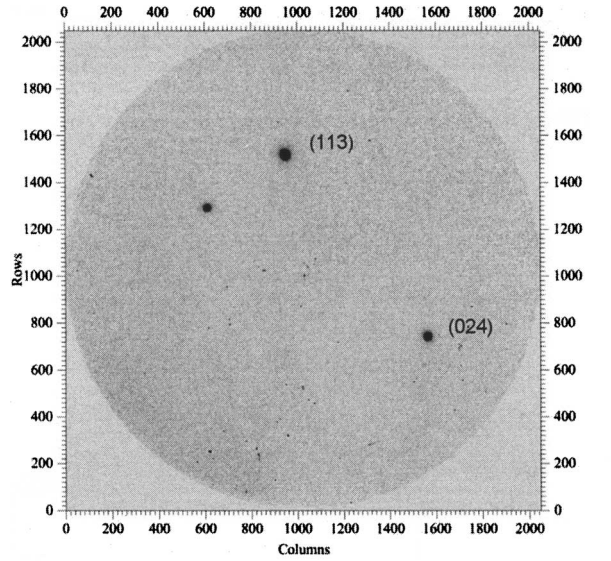

Figure 11. Typical diffraction pattern showing (113) and (024) reflections.

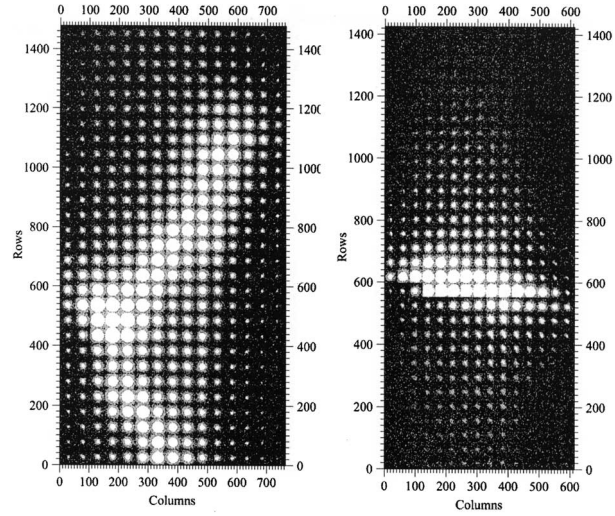

Figure 12. X-ray reflection intensities. (left) (113) reflection, (right) (024) reflection. The horizontal direction represents translation in steps of 1 μm. The vertical direction represents rotations by 0.1°.

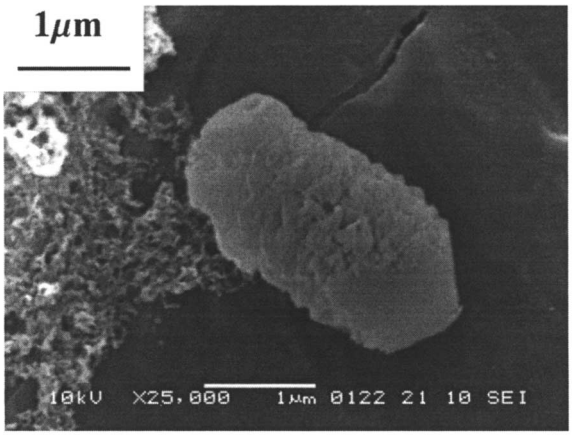

Figure 13. SEM of isolated pineal microcrystal on a Formvar covered TEM grid showing the multilayer structure.

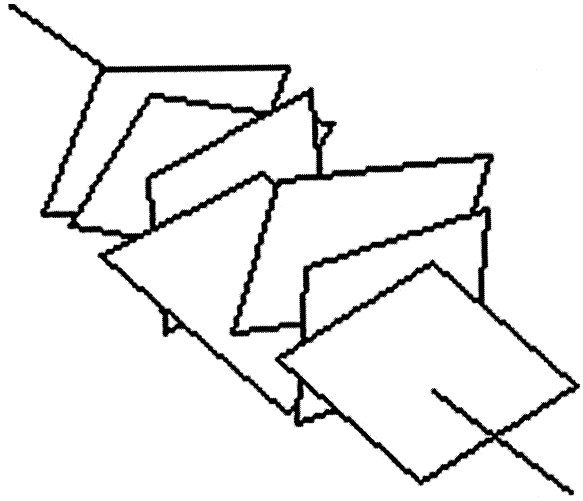

Figure 14. Schematic drawing showing a possible texture structure of microcrystals.

monocrystalline ones (Figure 13). The microcrystals, which have no phosphorus, do not have any relationship to the hydroxyapatite concretions [3]. The HRTEM and SEAD coupled with the Raman spectroscopy clearly show that the microcrystals are calcite and prove the presence of a new form of biomineralization in the human brain. Organic material appears on the surface of the biomineralized microcrystals as verified by additional non-calcite lines in the Raman spectra and the sulfur studies at the ESRF.

Reasons for the formation of the crystals and their possible biological significance are not known at present. However, the microimaging data and SEM photographs such as the multilayered structure in Figure 13 suggest a growth mechanism for the crystals. The crystal appears to have a texture consisting of a stack of thin rhombohedrons with their flat faces normal to the long axis of the crystal. A sketch of this type of structure is shown in Figure 14. The sharp edges and rough body could be explained in this way. Mineralogical calcite frequently exhibits complex structures [16]. The texture observed in the microcrystals could lead to symmetry breaking because of structural and/or stress gradients. This could result in the existence of properties normally associated with noncentrosymmetric crystals such as SHG and piezoelectricity.

The microcrystals bear a striking resemblance to the calcite crystals that form the otoconia of the inner ear. Otoconia have been studied extensively in a number of species, including human beings [17–19]. Their growth stages pass through ovoid, rhombohedral and cylindrical forms in a manner similar to those of the pineal microcrystals. The structure and chemical composition of the microcrystals are also similar to those found in the biomineralized crystals of sea urchin spines and sponge spicules [20].

We believe that the presence of two different crystalline compounds in the same organ is biologically significant. It is important to note that the calcite in otoconia has been shown to exhibit piezoelectricity [21], a property normally forbidden by crystallographic symmetry. Our current research is focused on direct measurements of possible piezoelectricity in the pineal calcite crystals and a consequent biological transducer mechanism.

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